Koisio technology-produced water significantly decreases lipopolysaccharide-induced inflammatory responses in microglial cell cultures

Yinghui Men, Weihai Ying*

Med-X Research Institute and School of Biomedical Engineering

Shanghai Jiao Tong University, Shanghai 200030, P.R. China

*: Corresponding author

Weihai Ying

School of Biomedical Engineering and Med-X Research Institute

Shanghai Jiao Tong University

Shanghai 200030, P.R. China

E-mail: weihaiy@sjtu.edu.cn

Abstract

Inflammation is a critical pathological factor in a number of diseases. Our previous study showed that Koisio technology-produced water (KW) significantly decreased the levels of inflammation and inflammation-induced damage in the mouse model of Dextran Sulfate (DSS)-induced ulcerative colitis. Since there are various types of inflammation, in this study we used a widely used cell culture model of neuroinflammation - lipopolysaccharide (LPS)-induced activation of microglial cell cultures – to determine if KW can also decrease neuroinflammation in this cell culture model. Our study found significantly lower mRNA levels of three pro-inflammatory cytokines including IL-1b, IL-6 and TNF-a in LPS-treated BV2 microglial cultures cultured in KW-based media, compared to those in the LPS-treated cells cultured in normal cell culture media. The KW-based cell culture media also significantly attenuated LPS-induced increases in the NO levels of the cells. Moreover, the KW-based cell culture media significantly attenuated LPS-induced increases in the oxidative stress levels of the cells. Our Western blot assays further showed that the KW-based cell culture media significantly attenuated LPS-induced increase in the level of nuclear NF-kB p65 in the BV2 cells. Collectively, our study has indicated that KW-based cell culture media significantly attenuated LPS-induced inflammatory responses of BV2 microglial cultures, at least partially by attenuating the LPS-induced increase in the level of nuclear NF-kB in the cells. This finding has further raised the possibility that KW may produce inhibitory effects on several types of inflammatory responses.

Keywords: Inflammation; Microglia; Koisio water; LPS; NF-kB

Introduction

Inflammation is a key pathological factor of multiple major diseases, which produces tissue injury by such processes as generating oxidative stress and a number of cytokines [1-4]. It is of important scientific and clinical significance to discover novel approaches for inhibiting inflammatory processes. Due to the escalating medical cost in many countries, it is also important to discover economical approaches to inhibit inflammatory responses.

Water is a major component of life, which occupies approximately 70 % of the space of human body. The quality of drinking water can produce significant impact on human health [5, 6]. Due to the significance of drinking water, it is critical to invent novel strategies to modulate the properties of water, so as to discover novel biological functions of water. It has been reported recently that through certain physical modulations, a type of pure water that contains numerous ultra-small nanobubbles has significant antioxidant effects, in which the ultra-small nanobubbles play key roles in the antioxidant effects [7].

Our previous study has shown that Koisio technology-produced water (KW) significantly decreased the levels of inflammation and inflammation-induced damage in the mouse model of DSS-induced ulcerative colitis [8], indicating that KW can inhibit certain types of inflammatory responses. Due to the critical scientific and clinical significance of inflammation, it is necessary to further determine if KW may also inhibit other types of inflammation. Therefore, in this study, we used a widely used cell culture model of neuroinflammation - LPS-induced activation of microglial

cell culture [9] – to determine if KW can also inhibit the LPS-induced neuroinflammation in microglial cell cultures. We also conducted studies to determine the mechanism underlying the effects of KW on the inflammatory responses.

Materials and Methods

Materials

All chemicals were purchased from Sigma (St. Louis, MO, USA) except where noted. KW was produced by the technical experts of Shanghai Koisio Food Industry Co. (Shanghai, China) according to standard procedures.

Methods

Cell culture models

Microglial (BV2) cell lines were purchased from the Chinese Academy of Sciences Cell Bank. BV2 cells were grown in KW-based DMEM or ddH_2O-based DMEM, both of which were supplemented with 10% FBS (100-500 GEMINI), 100U/mL penicillin, and 0.1 mg/mL streptomycin (15140122, Gibco) at 37 °C in humidified 5% CO_2 atmosphere. When the cell density reached 60% - 80%, the cells were treated with LPS for 24 h.

Production of cell culture media

The culture media were prepared according to the manufacturer's instructions. In brief, 13.5 g of DMEM powder (12800017, Gibco) was added to 950 mL sterilized purified water or KW, subsequently 1 N HCl was used to adjust the pH to around 4.1 - 4.2, followed by transferring 47.5 mL of media to a 50 mL centrifuge tube. Approximately 1.5 mL of 7.5 % $NaHCO_3$ solution was added to each 50 mL tube to adjust the pH to 7.2 – 7.4. Finally, sterile syringe filters with a 0.22 μm pore size (BE-PES-22, Biosharp) were used to filter the culture media.

Real-Time PCR

The real-time PCR assays were conducted as described previously [10, 11]. Total RNA was extracted by FastPure Cell/Tissue Total RNA Isolation Kit (Vazyme, Nanjing, China) from BV2 cells. Five hundred ng of total RNA was reverse-transcribed to cDNA using HiScript IV 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China). The parameters for reverse transcription were set as follows: 50°C for 5 min, then 85°C for 5 s. Quantitative RT-PCR was performed by using ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China) and the following primers:

IL-1β primers: sense 5′-TTGACGGACCCCAAAAGATG-3′, anti-sense 5′-AGAAGGTGCTCATGTCCTCA-3′;

IL-6 primers: sense 5′-TCTATACCACTTCACAAGTCGGA-3′, anti-sense 5′-GAATTGCCATTGCACAACTCTTT-3′;

TNF-α primers: sense 5′-TCTCATCAGTTCTATGGCCC-3′, anti-sense 5′-GGGAGTAGACAAGGTACAAC-3′;

GAPDH primers: sense 5′-CAACTTTGGCATTGTGGAAGG-3′, anti-sense 5′-ACACTTTGGGGGTAGGAACAC-3′.

PCRs were performed according to the following procedure: After denaturing at 95°C for 10 min, 40 cycles of 95°C for 10 s, 60°C for 10 s and 72°C for 10 s. The data were analyzed by using the comparative threshold cycle method, and the results were expressed as fold difference normalized to the level of GAPDH mRNA.

Determinations of intracellular ROS levels

For Dichlorofluorescin (DCF) assay, 2,7-Dichlorofluorescin diacetate (DCFH-DA, Beyotime, China), a reactive oxygen species-specific fluorescent probe, was used to measure total intracellular ROS levels. After BV2 cells were treated with 1 μg/mL LPS for 24 h, the cells were incubated with 20 μM DCFH-DA dissolved in DMEM without fetal bovine serum (FBS) for 30 min at 37°C in an incubator. After 3 times washes with PBS, the cells were analyzed by flow cytometry (FACS Aria; Becton Dickinson, Heidelberg, Germany) to detect the mean fluorescence intensity (MFI) with

an excitation wavelength of 488 nm and emission wavelength of 525 nm.

Nitric Oxide Assay

A NO Test Kit (Beyotime, Jiangsu, China) was used to determine the level of NO, according to the manufacturer's instructions. Cell culture medium, Griess reagent I, and Griess reagent II were mixed, followed by reading the absorbance at 540 nm using a microplate reader. The result was calculated by normalizing the concentrations of nitrite in the samples to concentrations of the standards, and concentrations of proteins which were determined by the bicinchoninic acid (BCA) assay.

Western blot assays

Nuclear and cytoplasmic proteins were extracted by the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, Jiangsu, China). In brief, after BV2 cells were treated with 1 μg/mL LPS for 24 h, cells were washed with PBS and scraped off by cell scrapers. For the 12-well plate, 200 μL of cytoplasmic protein extraction reagent A (with 1 mM phenylmethanesulfonyl fluoride) was added to each well. After that, the lysates were collected and strongly vortexed for 5 s to ensure cell precipitation was completely dispersed. The lysates were placed in an ice bath for 10 mins and 10 μL of cytoplasmic protein extraction reagent B was added, followed by 5 s strong vortex,

ice bath 1 min, 5 s strong vortex and centrifugation at 12,000 g for 5 mins at 4°C. The supernatant was cytoplasmic proteins. To extract nuclear protein, 50 μL of nuclear protein extraction reagent (with 1 mM phenylmethanesulfonyl fluoride) was added to the pellet, followed by 30 s strong vortex in an ice bath every 2 mins for total 30 mins. Finally, the lysates were centrifuged at 12,000 g for 10 mins at 4°C and the supernatant was nuclear protein.

After the extraction of nuclear and cytoplasmic protein, the content of protein was determined using the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, Illinois, United States), 20 μg of total protein was electrophoresed through a 10% sodium dodecyl sulfate-polyacrylamide gel and then transferred to 0.45-μm nitrocellulose membranes. The blots were incubated overnight at 4°C with primary antibodies. The antibody dilutions were as follows: anti-NF-κB p65 antibody (1:4,000, Abcam, United States), anti-Actin antibody (1:1,000, Santa Cruz, United States). Subsequently, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody in TBST containing 1% BSA at room temperature for 1 h. The intensities of the bands were quantified by densitometry using Gel-Pro Analyzer (Media Cybernetics, Silver Spring, United States).

Statistical Analysis

Data were presented as mean ± SEM and analyzed by one way analysis of variance (ANOVA) followed by Student-Newman-Keuls *post hoc* test except where

noted. *P* values less than 0.05 were considered statistically significant.

Results

1. KW-based cell culture media significantly attenuated LPS-induced increases in the levels of three pro-inflammatory cytokines in BV2 microglial cell cultures

IL-1β, IL-6 and TNF-α are important pro-inflammatory cytokines [12, 13]. We found that LPS induced significant increases in the mRNA levels of IL-1β, IL-6 and TNF-α in the BV2 cells cultured in normal cell culture media (**Figs. 1A, 1B, 1C**). In contrast, the mRNA levels of these cytokines were significantly lower in the LPS-treated cells cultured in the KW-based media (**Figs. 1A, 1B, 1C**).

2. KW-based cell culture media significantly decreased LPS-induced NO production in BV2 microglial cell cultures

LPS induced a significant increase in the NO level in the cells cultured in normal cell culture media (**Fig. 2**). The level of NO was significantly lower in the LPS-treated cells cultured in the KW-based media, compared to that in the cells cultured in normal cell culture media (**Fig. 2**).

3. KW-based cell culture media significantly attenuated LPS-induced increases in the levels of oxidative stress in BV2 microglial cell cultures

Our FACS-based assays showed that LPS induced significant increases in the DCF levels of the BV2 cells cultured in normal cell culture media (**Fig. 3**), which is an indicator of intracellular ROS levels. In contrast, the oxidative stress levels were

significantly lower in the LPS-treated cells cultured in the KW-based media, compared to those in the LPS-treated cells cultured in normal cell culture media (**Fig. 3).**

4. KW-based cell culture media significantly attenuated LPS-induced increase in the level of nuclear NF-kB p65 in BV2 microglial cell cultures

Our Western blot assays showed that LPS induced a significant increase in the level of nuclear NF-kB p65 in the BV2 cells cultured in normal cell culture media, which was significantly decreased in the BV2 cells cultured in the KW-based cell culture media (**Fig. 4A**). In contrast, there was no significant difference between the level of the cytoplasmic NF-kB p65 in the LPS-treated BV2 cells cultured in normal cell culture and that of the LPS-treated cells cultured in the KW-based cell culture media (**Fig. 4B).**

Discussion

The major findings of this study include: First, compared to normal cell culture media, KW-based cell culture media led to significantly attenuated increases in the levels of the three important pro-inflammatory cytokines, including IL-1b, IL-6 and TNF-a, in the LPS-treated BV2 microglial cell cultures; second, compared to normal cell culture media, KW-based cell culture media produced significantly attenuated increases in the NO levels in the LPS-treated BV2 microglial cell cultures; third, our study showed that KW can also significantly attenuate the LPS-induced oxidative stress of the cells; and fourth, our Western blot assays showed that the KW-based cell culture

media significantly attenuated LPS-induced increase in the level of nuclear NF-kB p65 in the BV2 cells. Collectively, our study has indicated that KW is capable of decreasing LPS-induced neuroinflammation in the microglial cell cultures, at least partially by attenuating the LPS-induced increase in the level of nuclear NF-kB p65 in the cells.

Neuroinflammation is a crucial pathological factor in multiple major neurological diseases, including stroke, Alzheimer's disease, Parkinosn's disease and multiple sclerosis [14]. LPS-induced microglial activation is a widely used cell culture model for neuroinflammation. Our current study has indicated that KW can significantly decrease the neuroinflammatory responses, suggesting that KW may be capable of inhibiting the neuroinflammatory responses in certain neurological disorders. It is warranted to conduct future studies to determine the effects of KW on the animal models of neurological diseases. Moreover, it is necessary to investigate the mechanisms underlying the KW-produced inhibition of neuroinflammation.

Our previous study has shown that KW can significantly decrease the inflammatory responses and inflammation-induced tissue damage in the mouse model of DSS-induced ulcerative colitis [8]. Our current study has provided evidence suggesting the mechanisms underlying the beneficial effects of KW in the animal model study: KW can markedly attenuate the inflammatory responses in LPS-induced microglial cell cultures, suggesting that KW may inhibit the activation of immune cells in the mouse model of ulcerative colitis. Since the activation of such immune cells as neutrophils can lead to increased release of myeloperoxidase (MPO) and eosinophil peroxidase (EPO), KW may lead to the attenuated increases in the levels of MPO and

EPO by inhibiting the activation of the immune cells.

NF-kB plays key roles in activation of inflammatory responses [15]. Our current study has shown that the KW-based cell culture media significantly attenuated LPS-induced increase in the level of nuclear NF-kB p65 in BV2 microglial cell cultures. This finding has indicated that KW produces inhibitory effects on LPS-induced inflammatory responses at least partially by inhibiting NF-kB. It is warranted to further investigate the mechanisms underlying the effects of KW on NF-kB.

Water is a key component of life. Therefore, searches for novel strategies to improve the healthy effects of water are critical for inventing economic and effective approaches to modulating inflammation. In our current study, we provided further evidence suggesting that KW can inhibit neuroinflammation, in addition to its inhibitory effects on inflammation in the mouse model of ulcerative colitis. Due to the critical roles of inflammation in a number of diseases as well as pre-disease state, our study has suggested that future studies regarding the effects of KW on inflammation are warranted.

References:

[1] W. Xia, J. Han, G. Huang, and W. Ying, "Inflammation in ischaemic brain injury: Current advances and future perspectives," *Clinical and Experimental Pharmacology and Physiology,* vol. 37, no. 2, pp. 253-258, 2010/02/01 2010, doi: https://doi.org/10.1111/j.1440-1681.2009.05279.x.

[2] C. I. Diakos, K. A. Charles, D. C. McMillan, and S. J. Clarke, "Cancer-related inflammation and treatment effectiveness," *The Lancet Oncology,* vol. 15, no. 11, pp. e493-e503, 2014, doi: 10.1016/S1470-2045(14)70263-3.

[3] M. T. Heneka *et al.*, "Neuroinflammation in Alzheimer's disease," *The Lancet Neurology,* vol. 14, no. 4, pp. 388-405, 2015, doi: 10.1016/S1474-4422(15)70016-5.

[4] M. G. Tansey, R. L. Wallings, M. C. Houser, M. K. Herrick, C. E. Keating, and V. Joers, "Inflammation and immune dysfunction in Parkinson disease," *Nature Reviews Immunology,* vol. 22, no. 11, pp. 657-673, 2022/11/01 2022, doi: 10.1038/s41577-022-00684-6.

[5] X. Liu, Z. Pei, Z. Zhang, Y. Zhang, and Y. Chen, "Associations of Boiled Water and Lifespan Water Sources With Mortality: A Cohort Study of 33,467 Older Adults," (in English), *Frontiers in Public Health,* Original Research vol. 10, 2022-June-27 2022, doi: 10.3389/fpubh.2022.921738.

[6] T. Feng, Z. Feng, Q. Liu, L. Jiang, Q. Yu, and K. Liu, "Drinking habits and water sources with the incidence of cognitive impairment in Chinese elderly population: The Chinese Longitudinal Healthy Longevity Survey," *Journal of*

Affective Disorders, vol. 281, pp. 406-412, 2021/02/15/ 2021, doi: https://doi.org/10.1016/j.jad.2020.12.044.

[7] J. Zheng *et al.*, "An antioxidation strategy based on ultra-small nanobubbles without exogenous antioxidants," *Scientific Reports,* vol. 13, no. 1, p. 8455, 2023/05/25 2023, doi: 10.1038/s41598-023-35766-5.

[8] Y. Men, M. Zhang, Y. Zhang, and W. Ying, "koisio Technology-Produced Water Significantly Decreased Inflammation and Multiple Injuries in Mouse Model of Dextran Sulfate Sodium Salt-Induced Acute Colon Inflammation," *chinaxiv,* 2022, doi: 10.12074/202212.00038V1.

[9] A. Skrzypczak-Wiercioch and K. Sałat, "Lipopolysaccharide-Induced Model of Neuroinflammation: Mechanisms of Action, Research Application and Future Directions for Its Use," *Molecules*, vol. 27, no. 17, doi: 10.3390/molecules27175481.

[10] B. Wang *et al.*, "NAD+ administration decreases doxorubicin-induced liver damage of mice by enhancing antioxidation capacity and decreasing DNA damage," *Chemico-Biological Interactions,* vol. 212, pp. 65-71, 2014/04/05/ 2014, doi: https://doi.org/10.1016/j.cbi.2014.01.013.

[11] C. Zhou, W. Shang, S.-K. Yin, H. Shi, and W. Ying, "Malate-Aspartate Shuttle Plays an Important Role in LPS-Induced Neuroinflammation of Mice Due to its Effect on STAT3 Phosphorylation," (in English), *Frontiers in Molecular Biosciences,* Original Research vol. 8, 2021-July-26 2021, doi: 10.3389/fmolb.2021.655687.

[12] H. Zhang and N. S. Dhalla, "The Role of Pro-Inflammatory Cytokines in the Pathogenesis of Cardiovascular Disease," *International Journal of Molecular Sciences*, vol. 25, no. 2, doi: 10.3390/ijms25021082.

[13] B. Möller and P. M. Villiger, "Inhibition of IL-1, IL-6, and TNF-α in immune-mediated inflammatory diseases," *Springer Seminars in Immunopathology,* vol. 27, no. 4, pp. 391-408, 2006/06/01 2006, doi: 10.1007/s00281-006-0012-9.

[14] K. Biswas, "Microglia mediated neuroinflammation in neurodegenerative diseases: A review on the cell signaling pathways involved in microglial activation," *Journal of Neuroimmunology,* vol. 383, p. 578180, 2023/10/15/ 2023, doi: https://doi.org/10.1016/j.jneuroim.2023.578180.

[15] D. Capece, D. Verzella, I. Flati, P. Arboretto, J. Cornice, and G. Franzoso, "NF-kB: blending metabolism, immunity, and inflammation," *Trends in Immunology,* vol. 43, no. 9, pp. 757-775, 2022, doi: 10.1016/j.it.2022.07.004.

Figure Legends:

Figure 1. KW significantly decreased LPS-induced increases in the mRNA levels of three pro-inflammatory cytokine levels of BV2 microglial cultures. BV2 cells were treated with 1 μg/mL LPS for 24 h. Subsequently real-time PCR assays were conducted to determine the mRNA levels of IL-1β, IL-6 and TNF-α in the LPS-treated BV2 cells cultured in normal cell culture media or KW-based media. N = 9. ***, $P < 0.001$ (one-way ANOVA test). ###, $P < 0.001$ (Student *t*-test).

Figure 2. KW significantly decreased LPS-induced NO production by BV2 microglial cultures. BV2 cells were treated with 0.1, 0.25, 0.5, 1 and 2 μg/mL LPS for 24 h. The nitrite levels in the media were detected using Griess reagent. N = 9. #, $P < 0.05$; ###, $P < 0.001$ (Student *t*-test).

Figure 3. KW showed a significantly greater capacity to decrease ROS levels in the LPS-induced BV2 microglial cells. BV2 cells were treated with 1 μg/mL LPS for 24 h. Subsequently FACS-based assays using DCFH as ROS probe were conducted, which showed that the intracellular ROS level in the cells cultured in KW-based media was significantly lower than that in the cells cultured in normal cell culture media. N = 6. **, $P < 0.01$; ***, $P < 0.001$.

Figure 4. Western blot assays showed that KW-based media attenuated LPS-induced increase in the level of nuclear NF-κB p65 in the BV2 cell nucleus. (A)

Western blot assays showed that KW-based media significantly attenuate LPS-induced increase in the level of nuclear NF-κB p65 in the BV2 cell nucleus. (B) Western blot assays did not show a significant difference between the level of cytoplasmic NF-κB of LPS-treated BV2 cells cultured in normal cell culture media and that of LPS-treated BV2 cells cultured in KW-based cell culture media. BV2 cells cultured in normal cell culture media or KW-based media were treated with 1 μg/mL LPS for 24 h. The nuclear and cytoplasmic NF-κB p65 protein was extracted and quantified by Western blot. N = 9. *, $P < 0.01$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 1.

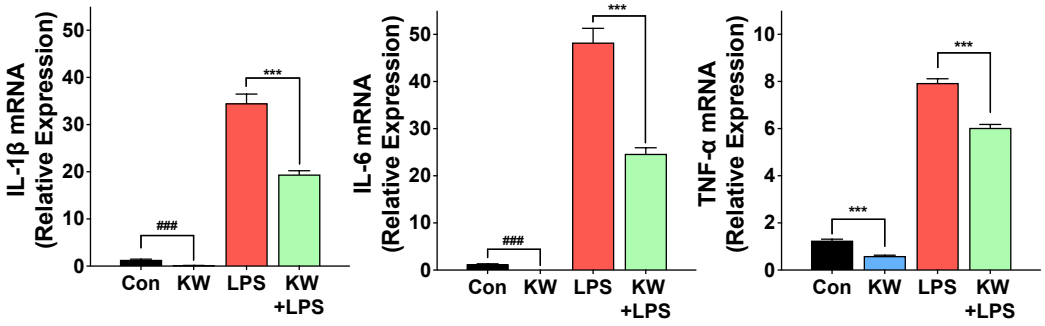

IL-1β mRNA (Relative Expression)
50
40
30
20
10
0
###
***
Con
KW
LPS
KW +LPS
IL-6 mRNA (Relative Expression)
50
40
30
20
10
0
###
***
Con
KW
LPS
KW +LPS
TNF-α mRNA (Relative Expression)
10
8
6
4
2
0
***
***
Con
KW
LPS
KW +LPS

Figure 2.

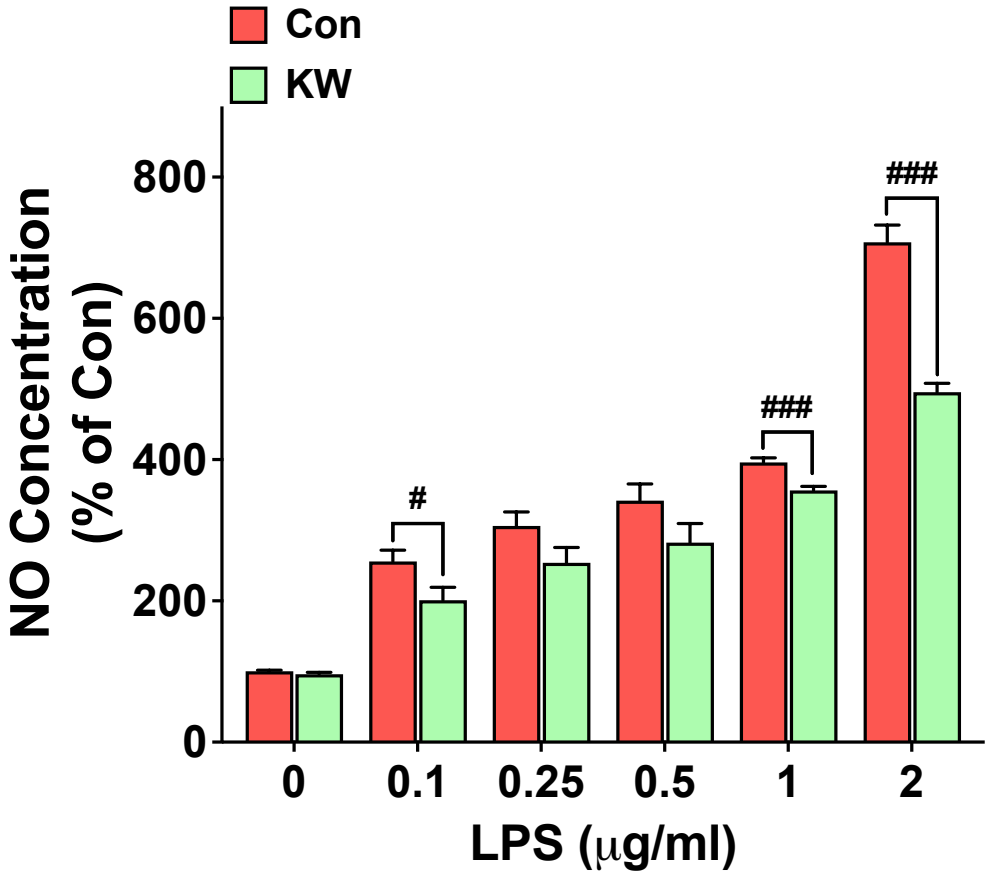

Con
KW
NO Concentration
(% of Con)
800
600
400
200
0
#
###
###
0
0.1
0.25
0.5
1
2
LPS (μg/ml)

Figure 3.

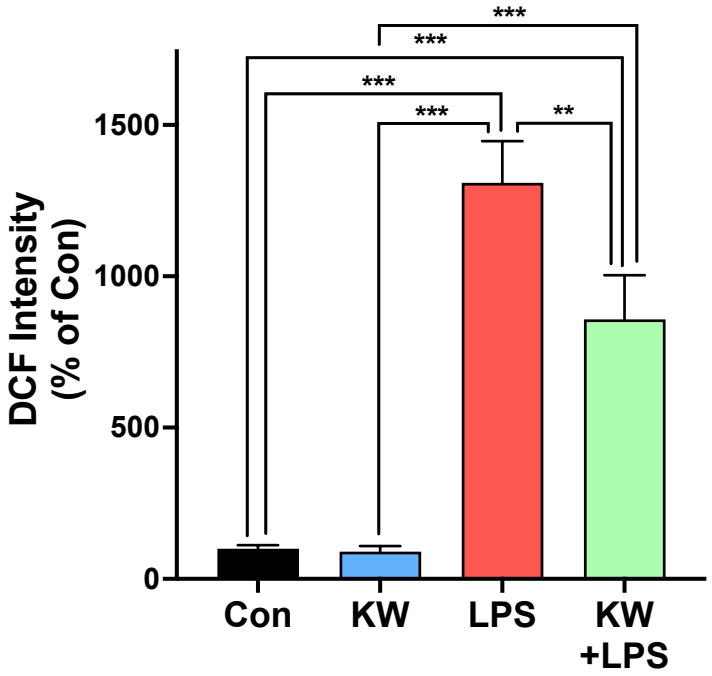
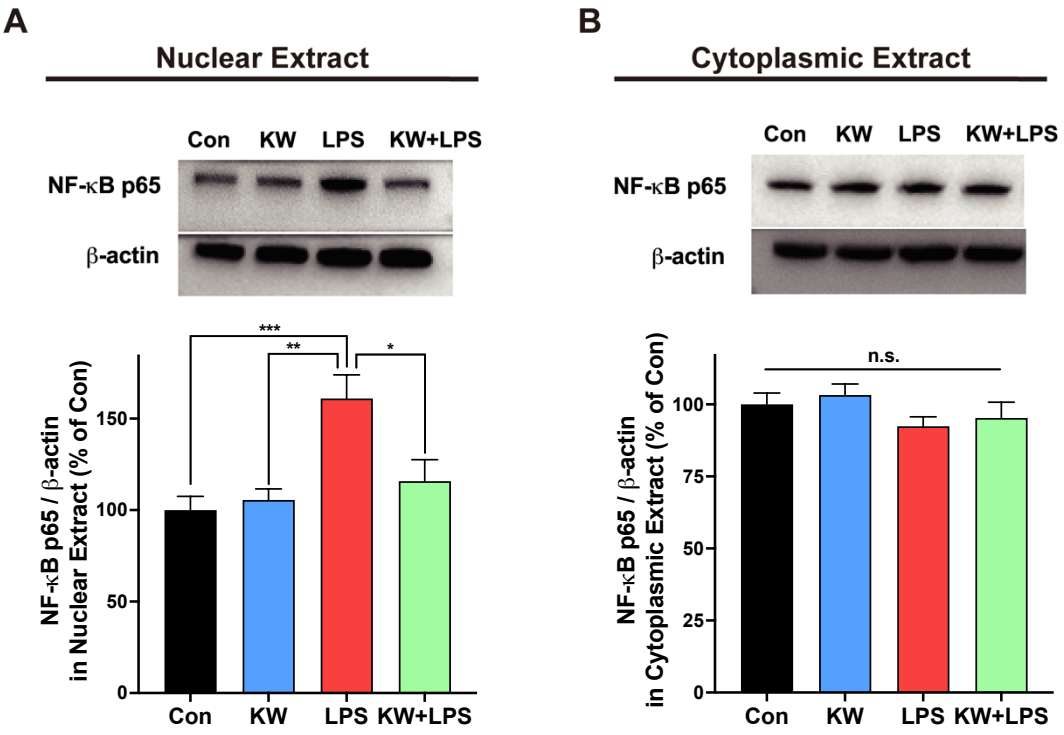


Figure 4.


A
Nuclear Extract
Con KW LPS KW+LPS
NF-κB p65
β-actin
***
**
*
NF-κB p65 / β-actin in Nuclear Extract (% of Con)
150
100
50
0
Con KW LPS KW+LPS
B
Cytoplasmic Extract
Con KW LPS KW+LPS
NF-κB p65
β-actin
n.s.
NF-κB p65 / β-actin in Cytoplasmic Extract (% of Con)
100
75
50
25
0
Con KW LPS KW+LPS